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A Single Amino Acid Substitution in SecY Stabilizes the Interaction with SecA*

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The SecYEG complex constitutes a protein conducting channel across the bacterial cytoplasmic membrane. It binds the peripheral ATPase SecA to form the translocase. When isoleucine 278 in transmembrane segment 7 of the SecY subunit was replaced by a unique cysteine, SecYEG supported an increased preprotein translocation and SecA translocation ATPase activity, and allowed translocation of a preprotein with a defective signal sequence. SecY(I278C)EG binds SecA with a higher affinity than normal SecYEG, in particular in the presence of ATP. The increased translocation activity of SecY(I278C)EG was confirmed in a purified system consisting of SecYEG proteoliposomes, while immunoprecipitation in detergent solution reveal that translocase-preprotein complexes are more stable with SecY(I278C) than with normal SecY. These data imply an important role for SecY transmembrane segment 7 in SecA binding. As improved SecA binding to SecY was also observed with the *prlA4* suppressor mutation, it may be a general mechanism underlying signal sequence suppression.

The components of the bacterial protein secretion pathway have originally been identified in *Escherichia coli* through both genetic (1, 2) and biochemical studies (3). The translocation reaction across the cytoplasmic membrane is mediated by an enzyme complex termed the translocase. The translocase holoenzyme is an assembly of integral membrane proteins, termed SecY (or PrlA) and SecE (PrlG), together with a peripheral ATPase termed SecA (PrlD). The SecYE complex is homologues to the eukaryotic Sec61p complex of the endoplasmic reticulum membrane (4) and both complexes appear to constitute a transmembrane protein conducting channel (5–7). The SecA protein is unique for bacteria, and for organelles evolutionary derived thereof (8). During cycles of ATP binding and hydrolysis SecA supports a stepwise translocation reaction (9, 10), coupled to cycles of membrane insertion and deinsertion at SecYE (11). An additional source of energy for the translocation reaction is the proton-motive force. The proton-motive force positively affects the unidirectionality of the translocation reaction (12), possibly by directly driving the translocation of preproteins in the absence of SecA (9) as well as by stimulating the SecA reaction cycle (13). Other proteinaceous factors involved in the translocation reaction are SecG (PrlH) (14) and a trimeric complex consisting of the SecD, SecF, and YajC proteins (15). SecG inverts its membrane topology concomitantly with the membrane cycling of SecA (16), whereas SecDFYajC stabilizes the membrane inserted state of SecA (17, 18). Both SecG and the SecDFYajC complex interact with the SecYE complex and stimulate translocation (15). For the efficient *in vitro* reconstitution of preprotein translocation, SecYEG is used as it is readily purified as a detergent-solubilized complex (19, 20). Stimulation of the translocation reaction by SecG has been demonstrated using the purified and reconstituted translocase (14, 21). Reconstituted SecYEG allows multiple rounds of translocation (22) as well as the integration of transmembrane segments into the lipid bilayer (23).

Genetic studies have identified mutations in translocase components that allow the correct cellular localization of preproteins carrying a defective signal sequence (24–28). How these *prl* mutations (for protein localization) suppress defective signal sequence recognition is yet unclear, but a direct restoration of the interaction between translocase and the signal sequence is unlikely. First, the number of *prl* alleles is too large to account for a single recognition event. Second, even (pre)proteins that lack the complete signal sequence are transported in strains carrying *prlA* (*secY*) or *prlG* (*secE*) suppressor mutations (29, 30). Alternatively, *prl* suppressor mutations may alter important enzymological events underlying the translocation reaction. Allele-specific synthetic lethality caused by combinations of *prlA* and *prlG* suppressor mutations suggest that they affect subunit interactions between SecY and SecE (31). Recently, it was shown that the *prlA4* suppressor mutation supports increased binding of SecA to translocation sites in the cytoplasmic membrane (32). This increased affinity for SecA leads to a decreased rejection of SecA or SecA-precursor complexes during translocation. Finally, *prlA* suppressors alter the translocation reaction less dependent on the proton-motive force (33).

In an effort to understand the nature and the dynamics of subunit interactions within the translocase, we employed cysteine-scanning mutagenesis to regions of SecY and SecE that contain, or are proximal to, clusters of *prl* suppressor mutations. Cysteine scanning mutagenesis has been used as a powerful technique to study structure-function relationships in membrane proteins, including the *E. coli* lactose permease LacY (34) and the eukaryotic multidrug transporter P-glycoprotein (35, 36). SecY contains *prlA* suppressor mutations that cluster mainly in transmembrane segment (TMS)[1] 2, TMS 7, and TMS 10, and in periplasmic loop 1 (P1) (37). Synthetic lethality between *prlG* and *prlA* suppressor mutations suggests interactions of SecE TMS 3 with SecY TMS 7 and 10, and between SecY P1 and SecE P2 (31). The replacement of consec-

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[1] The abbreviations used are: TMS, transmembrane segment; IMVs, inner membrane vesicles; octyl glucoside, *n*-octyl-β-D-glucopyranoside.

TABLE I
Plasmids

A synthetic secYEG operon behind the isopropyl-β-D-thiogalactoside-inducible trc promoter was used for the plasmid-derived overexpression of the SecYEG complex. All plasmids encoding single cysteine SecYEG were constructed via polymerase chain reaction mutagenesis, resulting in the indicated mutations.

Plasmid	Relevant characteristics	Mutations	Source
pET340	SecYEG tandem behind trc promotor		Ref. 20
pET349	His-tagged SecYEG in pET340		Ref. 20
pET602	Cysteine-less YEG in pET605		Ref. 38
pET605	pET340 with Δ*Hin*cII in secE	L60L (CTG->CTC)	Ref. 38
pET607	Cysteine-less YEG in pET610		Ref. 38
pET610	pET349 with Δ*Hin*cII in SecE	L60L (CTG->CTC)	Ref. 38
pET611	SecY TMS7 mutant 1 in pET602	V274C (GTA->TGT) G350G (GGT->GGA)	This study
pET612	SecY TMS7 mutant 2 in pET602	I275C (ATC->TGC) G350G (GGT->GGA)	This study
pET613	SecY TMS7 mutant 3 in pET602	P276C (CCG->TGT) G350G (GGT->GGA)	This study
pET614	SecY TMS7 mutant 4 in pET602	A277C (GCA->TGT) G350G (GGT->GGA)	This study
pET615	SecY TMS7 mutant 5 in pET602	I278C (ATC->TGC) G350G (GGT->GGA)	This study
pET616	SecY TMS7 mutant 6 in pET602	F279C (TTC->TGC) G350G (GGT->GGA)	This study
pET617	SecY TMS7 mutant 7 in pET602	A280C (GCT->TGT) G350G (GGT->GGA)	This study
pET618	SecY TMS7 mutant 8 in pET602	S281C (TCC->TGC) G350G (GGT->GGA)	This study
pET650	His-tagged SecY TMS7 mutant 5 in pET340		This study

utive residues by cysteines in SecY TMS 2 and SecE TMS 3 identified contacts at specific helical interfaces between these two protein regions and between neighboring SecE molecules (38). The latter interaction is dynamic and is modulated by conformational changes in the SecA protein. We now report on the cysteine mutagenesis of SecY TMS 7, which yielded a SecY molecule that supports increased translocation ATPase activity. This mutant possesses a unique cysteine at the position of isoleucine 278, a residue that is altered by several *prlA* suppressor mutations. Inner membrane vesicles (IMVs) or proteoliposomes containing SecY(I278C)EG not only supported increased translocation of normal preproteins, but also allowed translocation of a preprotein carrying a defective signal sequence. Binding studies further demonstrated that, like the PrlA4 suppressor, SecY(I278C) has a higher affinity for SecA than normal SecY. The latter permits efficient co-immunoprecipitation of translocase-preprotein complexes even without prior stabilization by a preprotein translocation intermediate. The data suggest an important role for SecY TMS 7 in SecA binding and support a model in which stabilization of the SecA-SecY interaction leads to increased translocation of normal preproteins concomitant with a reduced rejection of preproteins with a defective signal sequence.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibodies against OmpA were raised and selected by Prof. Dr. L. de Leij, Academic Hospital Groningen. Polyclonal antisera against purified SecY or SecA were obtained as described (20). Western blots were developed as films using chemiluminescence (Tropix, Bedford, MA). For densitometry a Dextra DF-2400T scanner (Dextra Technology Corp., Taipei, Taiwan) and SigmaScan/Image Software (Jandel Corp., San Rafael, CA) were used. DNA sequence analysis was performed on a Vistra DNA sequencer 725 using the automated Δtaq sequencing kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Protein A-Sepharose was from Amersham Pharmacia Biotech (Uppsala, Sweden), *n*-octyl-β-D-glucopyranoside (octyl glucoside) from Sigma, and *E. coli* phospholipids from Avanti Polar Lipids Inc. (Alabaster, AL).

Plasmids—All plasmids used for this study are described in Table I. The construction of plasmids that allow the overexpression of SecYEG, $(His)_6$-tagged SecYEG (20), or cysteine-less SecYEG (38) has been described previously. Cysteines were introduced in SecY TMS 7 by a two-step polymerase chain reaction mutagenesis. To facilitate the screening for correct mutants, cysteine mutagenesis was accompanied by the GGT → GGA (G350G) mutation, leading to the insertion of a Bsp*EI* restriction site. An amino-terminal $(His)_6$-tag on SecY(I278C) was obtained by cloning the *Nco*I/Bam*HI* fragment from pET615 (Table I) into Nco*I*/Bam*HI*-digested pET302 (20). All constructs were confirmed by sequence analysis.

Translocation Reactions—$(His)_6$-tagged SecYEG was purified and reconstituted into proteoliposomes as described (20), and other components of the translocation reaction were obtained as in Ref. 32. Concentrations of the different components are mentioned in the text or figure legends. Reactions were incubated at 37 °C in a total volume of 100 μl of translocation buffer (50 mM Hepes-KOH, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin, and 10 mM dithiothreitol) and stopped by chilling on ice and protease K treatment (10).

Immunoprecipitation—Proteoliposomes from two translocation reactions were collected by centrifugation (20 min, 120,000 × *g*), and solubilized in buffer C (1.25% (w/v) octyl glucoside, 0.3 mg/ml *E. coli* phospholipids, 20% (v/v) glycerol, 50 mM KCl and 50 mM Tris-HCl, pH 8.0) for 1 h on ice. Protein A-Sepharose slurry (10 μl) was incubated with 20 μl of antiserum diluted in 200 μl of buffer A for 1 h at 4 °C, washed, and mixed with the solubilized proteoliposomes. After 90 min of constant shaking at 4 °C, Sepharose beads were collected (3 min, 12,000 × *g*) and washed five times with 0.3 ml of buffer C. Bound proteins were eluted by incubation with 60 μl of SDS sample buffer for 10 min at 60 °C and separated from the Sepharose beads by centrifugation.

RESULTS

Identification of a Mutation in SecY That Supports Increased Translocation—As part of a larger cysteine-scanning mutagenesis study (38), unique cysteine residues were introduced in TMS 7 of SecY. To cover at least two turns of the putative α-helical structure, 8 residues in TMS 7 (Val^{274}-Ser^{281}) were mutagenized to cysteine residues (Fig. 1). Substitutions of two of these residues that face the same side of the helix, Val^{274} and Ile^{278}, have been reported to give rise to suppressor phenotypes (37). The mutant *secY* genes were cloned in pET602, a vector that allows overexpression of cysteine-less SecYEG, which is functionally indistinguishable from normal SecYEG (38). Although the expression of SecYEG was similar with all TMS 7 mutants (Fig. 2*A*), there was a pronounced increase in SecA ATPase activity with SecY(I278C)EG IMVs (Fig. 2*B*). Moreover, this resulted in increased translocation of the preprotein proOmpA (Fig. 2*C*). To test whether the increased activity of the SecY(I278C)EG complex affected its specificity, we examined the translocation of Δ8proOmpA, a variant precursor carrying a defective signal sequence due to the deletion of Ile^8 (41) that is efficiently translocated by PrlA4 IMVs (32). This precursor was transported only into the SecY(I278C)EG IMVs (Fig. 2*D*), demonstrating that the I278C mutation causes a loss of specificity for the signal sequence. Apparently, the introduction of cysteines at the other positions of SecY TMS 7, including Val^{274}, did not alter the activity or specificity of translocase.

Increased SecA Binding to SecY(I278C)EG—With the PrlA4 suppressor, which contains the F286Y substitution in TMS 7 and I408N in TMS 10, an increased affinity for SecA was observed as compared with normal PrlA (SecY). The difference in SecA binding is even larger upon the addition of ATP, which

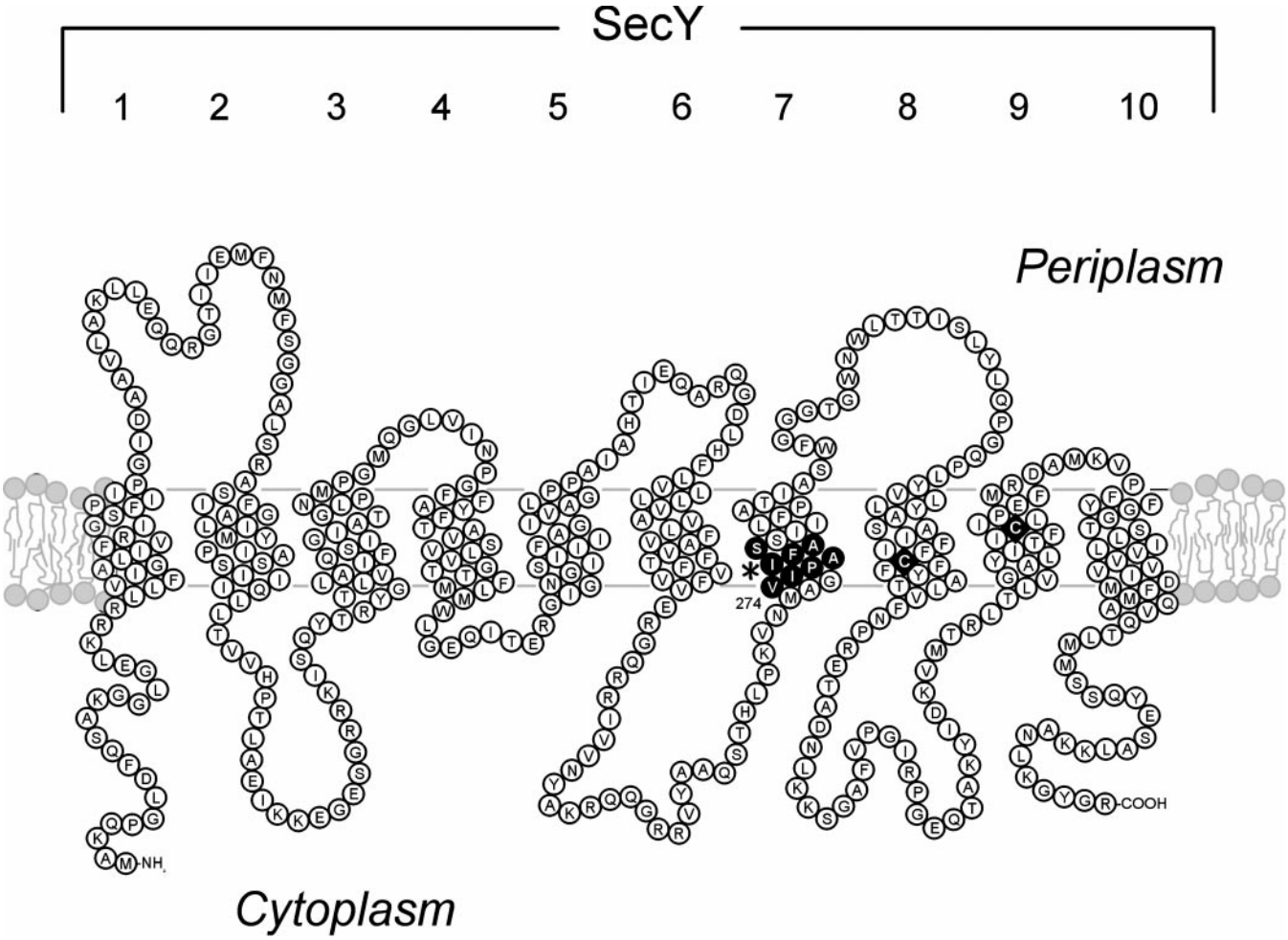

FIG. 1. **Localization of unique cysteines in SecY and SecE.** Amino acids were replaced by cysteines (*black circles*) in SecY TMS 7 using a cysteine-less SecYEG background. SecE and SecG are devoid of cysteines and the two endogenous cysteines of SecY (*black diamonds*) were replaced by serine residues (38). Positions of the replaced residues are based upon topology models of SecY (39, 40). The position of the I278C substitution in SecY TMS 7 is marked with an *asterisk*.

lowers the affinity, but to a much lesser extent with PrlA4 (32). Since SecY(I278C) allows translocation of Δ8proOmpA, we compared the binding of SecA to IMVs containing overproduced SecYEG, cysteine-less SecYEG or SecY(I278C)EG (Fig. 3). As expected from their similar activity (Fig. 2), the binding of SecA to SecYEG or cysteine-less SecYEG was nearly identical (Fig. 3*A, closed bars*) and was reduced to the same level in the presence of ATP (*open bars*). In contrast, the binding of SecA to SecY(I278C)EG IMVs was significantly higher and was only slightly reduced in the presence of ATP. Using a concentration range of SecA, we determined the affinity of SecA binding to the IMVs containing cysteine-less SecYEG (Fig. 3*B*) or SecY(I278C)EG (Fig. 3*C*) by Scatchard analysis (42). IMVs contained 2.1–2.4 μM/mg high affinity SecA-binding sites, a 25–30-fold increase as compared with IMVs harboring endogenous levels of SecYEG (80 nM/mg; Ref. 32). SecA binds to overproduced cysteine-less SecYEG with a K_d of 4 nM in the absence and a K_d of 16 nM in the presence of ATP. These affinities are somewhat higher, but comparable, to those observed with endogenous SecYEG, *i.e.* 7 nM without and 24 nM with ATP (32), and confirm that SecYEG is functionally overexpressed. Compared with cysteine-less SecYEG, the affinity of SecA binding to SecY(I278C)EG was 2.5-fold higher in the absence of ATP (K_d = 1.6 nM) and 5.7-fold higher in the presence of ATP (K_d = 2.8 nM). These data demonstrate that the SecY(I278C) mutation results in an increased affinity of the SecYEG complex for SecA, especially in the presence of ATP. With PrlA4, SecA binding occurs with a K_d of 1.4 nM in the absence and a K_d of 3.6 in the presence of ATP. The increased affinity leads to a decreased rejection of SecA-precursor complexes, and less dissociation of SecA during translocation (32). We propose that the same phenomenon is responsible for the increased translocation activity and lowered specificity of SecY(I278C).

Translocation Activity of Purified SecY(I278C)EG—To study the translocation activity of the purified SecY(I278C)EG complex, a $(His)_6$-tag was positioned at the amino terminus of SecY. The complex was then overexpressed, purified, and reconstituted as described previously (20). $(His)_6$SecY(I278C)EG proteoliposomes were compared with those reconstituted with the same amount of normal $(His)_6$SecYEG. As observed with IMVs, the proOmpA-stimulated ATPase activity was highest with SecY(I278C)EG proteoliposomes (Fig. 4*A*, *closed bars*). However, when Δ8proOmpA was used in the translocation reaction, hardly any stimulation of the SecA translocation ATPase activity was observed (Fig. 4*A*). Therefore, the amounts of translocated precursor were visualized by Western blotting using monoclonal antibodies against OmpA. Quantitative analysis of these blots demonstrated that after 20 min only a minor fraction (<0.5%) of the Δ8proOmpA was translocated in SecY(I278)EG proteoliposomes, as compared with normal proOmpA (about 25%) (Fig. 4, *B* and *C*, *closed symbols*). To demonstrate that the translocated protein was truly Δ8proOmpA, and not the result of an impurity with endoge-

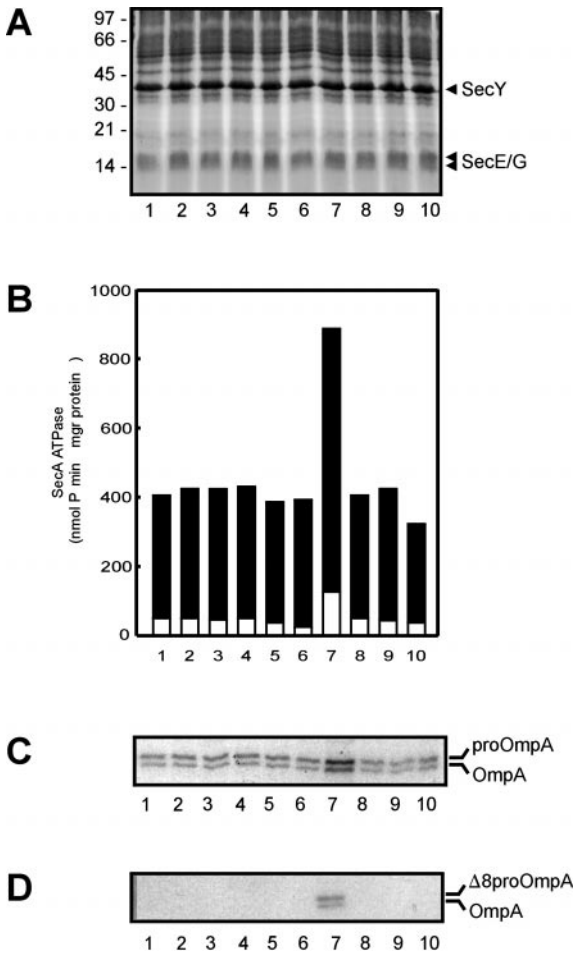

FIG. 2. **Overexpression and activity of the SecY TMS 7 cysteine mutants.** *A,* isolated IMVs containing overexpressed normal SecYEG (*lane 1*), cysteine-less SecYEG (*lane 2*), or SecYEG with unique cysteines at positions 274–281 of SecY TMS 7 (*lanes 3–10*) were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. *B,* SecA ATPase activity in the presence of urea-treated IMVs was measured in the absence (*open bars*) and presence (*black bars*) of proOmpA, as described previously (20). Data represent the average of two experiments, IMVs are numbered as in *A*. *C,* translocation of [^{35}S]methionine-labeled proOmpA with urea-treated IMVs (20 μg/ml) in the presence of SecA (10 μg/ml) and ATP (2 mM). Reactions (50 μl) were stopped after 20 min by chilling on ice and protease K treatment, yielding protease-protected proOmpA which is partially processed to OmpA by endogenous leader peptidase. *D,* translocation of [^{35}S]methionine-labeled Δ8proOmpA, carrying a defective signal sequence using the conditions described under *C*.

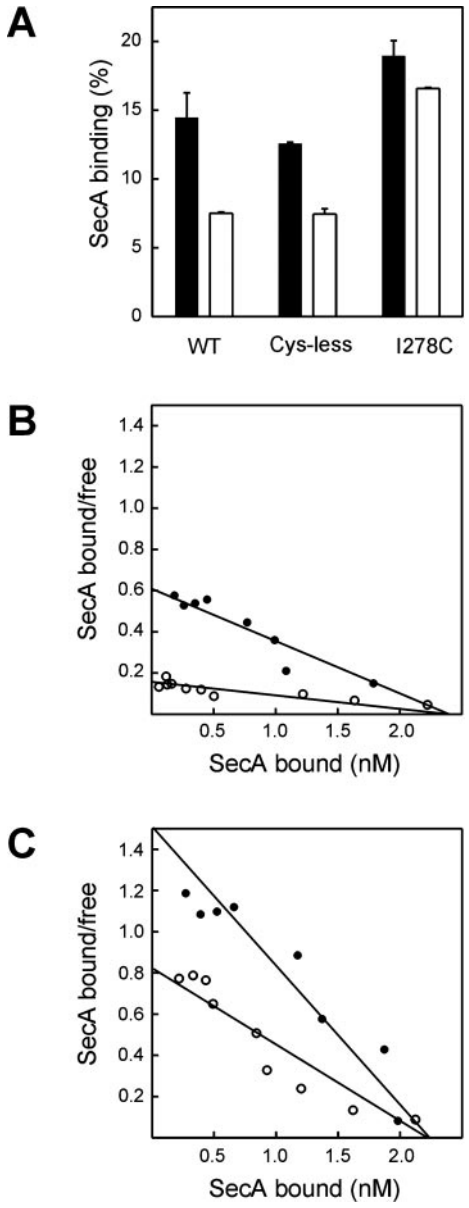

FIG. 3. **Increased SecA binding to SecY(I278C)EG.** *A,* SecA binding to IMVs containing overexpressed SecYEG (*WT*), cysteine-less SecYEG (*Cys-less*), or SecY(I278C)EG (*I278C*). Binding was determined using ^{125}I-labeled SecA (2 μg/ml) and urea-treated IMVs (10 μg/ml) in the absence (*filled bars*) and presence (*open bars*) of 2 mM ATP. *B,* Scatchard analysis of SecA binding (1–200 nM) to urea-treated IMVs (10 μg/ml) containing overexpressed cysteine-less SecYEG in the absence (*closed circles*) and presence (*open circles*) of 2 mM ATP. *C,* same as *B*, with IMVs containing overexpressed SecY(I278C)EG. All binding experiments were performed as described previously (32, 47). As no detectable background binding of SecA was observed, uncorrected data are shown.

nous proOmpA from the host strain used for purification, we repeated the experiment with *in vitro* synthesized and purified [^{35}S]methionine-labeled Δ8proOmpA (Fig. 4*D*). This clearly showed that purified SecY(I278C)EG allows the translocation of this defective precursor. The sensitivity of the autoradiograms (Fig. 4*D*) was somewhat higher than that obtained by Western blots (Fig. 4*C*) and revealed a minimal level of Δ8proOmpA translocation with normal SecYEG, confirming *in vivo* data (41).

In the absence of reducing agents, proOmpA is blocked for further translocation at the position of a disulfide-bond between two unique cysteine residues (Cys^{290} and Cys^{302}) in its carboxyl terminus (9, 43). In proteoliposomes, this results in the accumulation of a 31-kDa translocation intermediate (I_{31}) (Fig. 4*E*). This intermediate occupies the translocation sites and blocks them for a second round of translocation (Ref. 22 and data not shown). With the normal proteoliposomes, maximal I_{31} translocation was reached after 10 min, whereas proteoliposomes with SecY(I278C) accumulated maximal amounts within the first 5 min of the translocation reaction (Fig. 4*E*). SecY(I278C) did not allow the full-length translocation of oxidized proOmpA and thus differs in this respect from PrlA4 (33). The fast kinetics of the translocation reaction with SecY(I278C), as compared with normal SecY, is apparent from the initial rate of translocation (Fig. 4*B*) and the shorter time required to saturate the translocation sites with I_{31} (Fig. 4*E*). The experiments with proteoliposomes demonstrate that the SecY(I278C) mutation stimulates SecA- and ATP-driven translocation, and that this effect does not require proteinaceous factors other than the SecYEG complex. In addition,

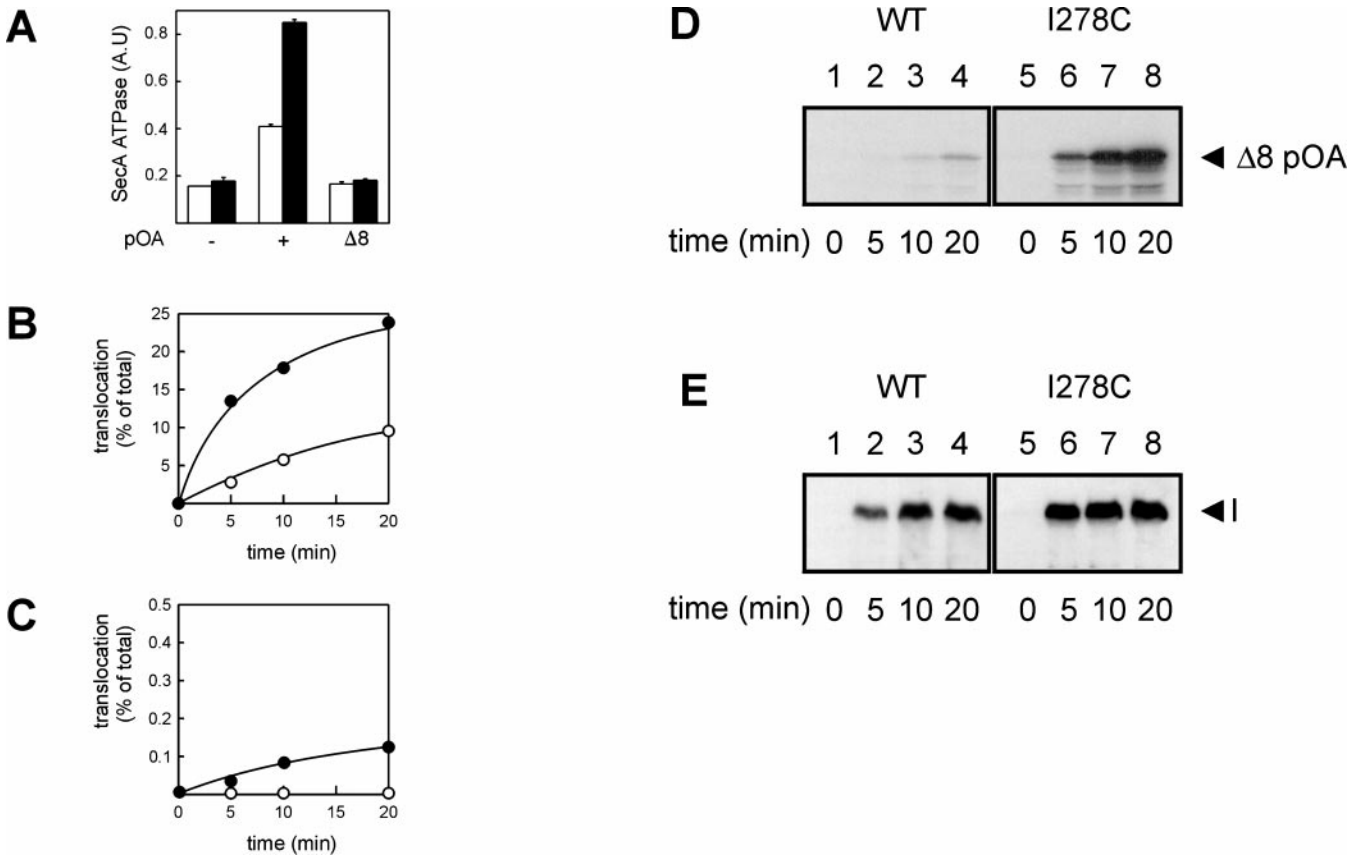

FIG. 4. **SecY(I278C) increases the activity of the translocase.** *A,* ATPase activities of 20 μg/ml SecA was measured after 20 min of incubation with 2 mM ATP and 4 μg/ml reconstituted purified SecYEG (*open bars*) or SecY(I278C)EG (*closed bars*) (4–6 μl of proteoliposomes), in the absence (−) or presence (+) of 10 μg/ml proOmpA or Δ8proOmpA (Δ8). *B,* translocation of proOmpA with reconstituted SecYEG (*open circles*) or SecY(I278C)EG (*closed circles*) was performed as in *A*, and stopped at the indicated times by chilling on ice and protease K treatment. Protease-protected proOmpA was visualized by immunoblotting using a monoclonal antibody against OmpA and quantitated by densitometrical analysis of films from chemiluminescent blots. *C,* same as *B*, with Δ8proOmpA. *D,* translocation of [^{35}S]methionine-labeled Δ8proOmpA into proteoliposomes containing SecYEG (*lanes 1–4*) or SecY(I278C)EG (*lanes 5–8*). *E,* immunoblot of protease-protected proOmpA after translocation in the absence of reducing agents, with SecYEG (*lanes 1–4*) or SecY(I278C)EG proteoliposomes. A disulfide bond between Cys^{290} and Cys^{302} in proOmpA results in the accumulation of translocation intermediate I_{31}.

SecY(I278C) enforces the translocation of Δ8proOmpA with purified translocase.

SecY(I278C) Stabilizes Translocase-Precursor Complexes—Co-immunoprecipitation was used to assay the stability of translocase-precursor complexes formed during ongoing translocation or at halted stages of the translocation reaction. Proteoliposomes were incubated with SecA and proOmpA in the absence of ATP (targeting of SecA and the precursor), the presence of ATP (ongoing translocation), or with ATP under oxidizing conditions (blocked translocation, yielding I_{31}). The proteoliposomes were then harvested by centrifugation and solubilized in the detergent octyl glucoside. Samples were immunoprecipitated with polyclonal antiserum against SecA or SecY, and co-precipitation of proOmpA was visualized by immunoblotting using a monoclonal antibody against OmpA (Fig. 5*A*). No, or only very little, interaction between proOmpA and either SecY or SecA was observed with normal translocase after incubation in the absence of ATP (*lanes 1* and *7*). After translocation under reducing conditions, a fraction of proOmpA was associated with SecY but not with SecA (*lanes 2* and *8*). Apparently, SecA has dissociated from these SecYEG-precursor complexes. Only after translocation of I_{31}, fully stable translocase-precursor complexes were formed (*lanes 3* and *9*). In contrast, SecY(I278C) translocase-precursor complexes were precipitated independent of the preincubation (*lanes 4–6* and *10–12*). Only immunoprecipitation with anti-SecYE serum yielded a significantly lowered amount of proOmpA after incubation in the absence of ATP (*lane 4*). Antibodies against SecY interfere with SecA binding (44, 45) and therefore may destabilize translocase. In conclusion, SecY(I278C) translocase-precursor complexes are more stable than their counterparts containing normal SecY. Wild-type translocase is, however, stabilized by the I_{31} translocation intermediate. This is consistent with experiments in IMVs that suggest a stable association of this intermediate with SecA at translocation sites (9).

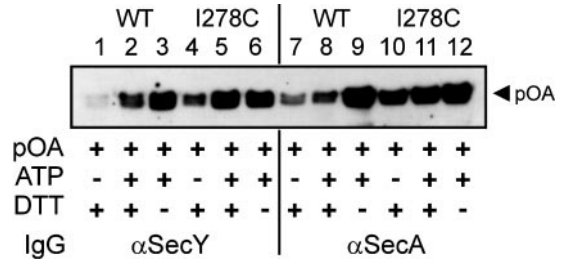

FIG. 5. **Co-immunoprecipitation of proOmpA with translocase.** SecYEG (*lanes 1–3* and *7–9*) or SecY(I278C)EG (*lanes 4–6* and *10–12*) proteoliposomes were incubated for 20 min with proOmpA and 2 mM ATP and/or 10 mM dithiothreitol, as indicated. After solubilization of the proteoliposomes with octyl glucoside, samples were immunoprecipitated with antibody against SecY (*lanes 1–6*) or SecA (*lanes 7–12*). Co-precipitation of proOmpA was analyzed by immunoblotting using a monoclonal antibody against OmpA.

DISCUSSION

The *E. coli* translocase is composed of the SecA ATPase bound to a transmembrane protein conducting channel with SecY and SecE as core components (19), and with SecG as an additional subunit (14, 21). The identification and reconstitution of its minimal constituents (19–21) have made translocase an intriguing model to study subunit dynamics in a membrane protein complex. To allow site-directed labeling of functionally important regions in translocase and to detect specific intermolecular contacts, we have employed cysteine mutagenesis of regions in SecY and SecE that contain clusters of *prlA* or *prlG* suppressor mutations, respectively (31, 37, 38). From the single cysteine mutants at positions 274–281 of SecY TMS 7, the I278C substitution resulted in an increased translocation activity and gave rise to *in vitro* defective signal sequence suppression, as measured by the translocation of Δ8proOmpA. Previously, *prlA* suppressor mutations have been identified that lead to substitutions of I278 for Ser (*prlA202, 203, 204,* and *207*), Asn (*prlA208*), or Thr (*prlA303*) residues (37). It thus seems that Ile^{278} is a hot spot for such suppressor mutations. SecY(V274C) did not alter the activity and specificity of translocase, although *prlA* suppressor mutations have been identified that result in a V274G substitution (*prlA1*, *2*, *5* and *201*; Ref. 37). Apparently, the amino acid substitutions that give rise to *prl* suppression depend not only on the position but also on the nature of the substituted amino acid. We have also constructed plasmids that allowed co-overexpression of the SecY mutants with cysteines at positions 105–109 of SecE TMS 3. Although synthetic lethality was observed between *prlA208* (I278N) and *prlG1* (L108R) (31), none of the combined mutants yielded cross-links between SecY and SecE upon oxidation (data not shown). This implies that synthetic lethality does not necessarily result from a direct interaction between two amino acids.

One of the earliest identified *prlA* suppressor mutations is *prlA4* (24). Its suppressor phenotype is caused by the I408N substitution in SecY TMS 10, but this mutation is generally accompanied by the F286Y substitution in TMS 7 or, with *prlA6*, S188L in TMS 5 (37, 46, 47). The apparently unavoidable occurrence of secondary mutations may reflect a detrimental effect of the I408N substitution on the *E. coli* cell. SecA binds to PrlA4 with an increased affinity, and this results in a decreased rejection of SecA and the preprotein at the onset of translocation (32). We now report on a similar phenomenon with SecY carrying the I278C amino acid substitution in TMS 7 in a cysteine-less background. This mutant supports an increased translocase activity and translocates Δ8proOmpA, carrying a defective signal sequence. As the activity of cysteine-less SecYEG is indistinguishable from normal SecYEG (this study and Ref. 38), the I278C mutation appears solely responsible for the observed phenotype (see Fig. 2*D*). Using affinity blotting, the amino-terminal half of SecY was detected as a binding site for SecA (48). The increased affinity for SecA caused by this I278C substitution, however, indicates that SecY TMS 7 also serves as a site of interaction with SecA. Alternatively, this mutation affects binding of SecA to the amino-terminal half of SecY. Our data support a model in which *prlA* suppression is the result of improved binding between SecA and SecY. This will optimize the translocation of normal preproteins due to a better targeting of SecA to SecYEG and less dissociation of the translocase components during ATP-driven translocation. At the same time, it lowers the proofreading activity of translocase as SecA carrying a defective preprotein is less easily rejected from translocation sites at the onset of translocation, likely upon the binding of ATP. Since proton-motive force-driven translocation is prevented by the presence of SecA (9), an increased affinity for SecA may explain why PrlA suppressors render the translocation reaction proton-motive force-independent (33).

The stabilization of translocase-precursor complexes by SecY(I278C) during translocation was directly demonstrated by co-immunoprecipitation. With normal SecY, a soluble translocase-precursor complex required the presence of a stable translocation intermediate I_{31}. No complexes between SecA or SecY with the precursor were observed after incubation in the absence of ATP, and SecA readily dissociated from the complex during an ongoing translocation reaction. With SecY(I278), translocase-preprotein complexes were completely stable after incubation under translocating conditions, as compared with complexes with trapped I_{31}. Incubation in the absence of ATP yielded complexes that were susceptible for dissociation by an antibody against SecY. This suggests that the SecYEG channel alters its conformation during translocation, rendering the interaction with SecA and the preprotein more stable. This conformational change may involve subunit rearrangements, or channel "opening," as has been observed with the Sec61p complex during translocation (49, 50).

Whereas increased SecA binding is a clear phenotype of both PrlA4 and SecY(I278C), they are functionally different in two aspects. First, PrlA4 allows translocation of a disulfide bonded loop of 10 amino acids in the mature region of proOmpA (33), whereas SecY(I278C) does not (Fig. 4*E*). Second, PrlA4 supports increased translocation with a lowered SecA ATPase activity (32), whereas with SecY(I278C), the increased translocation is accompanied by a concomitant increase in the rate of ATP hydrolysis by SecA. The affinity of PrlA4 and SecY(I278C) for SecA is hardly different and we therefore hypothesize that PrlA4 and SecY(I278C) differ mechanistically. Suppressor mutations in SecY TMS 10 appear to strongly affect the interaction with SecE TMS 3 (31, 37). As conformational changes in SecE TMS 3 and SecA membrane cycling are interrelated (38), the I408N mutation in PrlA4 may slow the SecA reaction cycle due to an altered interaction with SecE. A slowed ATPase activity has been proposed as a mechanism for *prlD* (*secA*) suppression by increasing the lifetime of SecA-preprotein complexes during translocation (27). With PrlA4, improved SecA binding to the SecYEG complex at the same time makes translocation highly efficient. We propose that SecY(I278C) is a milder suppressor than PrlA4 because it does not affect the SecA reaction cycle. Extensive biochemical analysis of other Prl suppressors will unravel more of the mechanistic aspects underlying signal sequence recognition and the activity of translocase.

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